Biointerface Research in Applied Chemistry
Open-Access Journal (ISSN: 2069-5837)

Article
Volume 13, Issue 4, 2023, 322
https://doi.org/10.33263/BRIAC134.322

Comparative Performance of Two Immunochromatographic Tests for the Rapid Detection of PCR Confirmed, Carbapenemase Producing-*Enterobacterales*

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Received: 15.06.2022; Accepted: 30.07.2022; Published: 11.09.2022

Abstract: The Gram-negative bacilli (GNB) tend to dominate the infectious pathology, often due to multidrug-resistant (MDR) strains and evolving with severe, complicated, and difficult-to-treat clinical forms. This study aimed to investigate by phenotypic and genotypic assays a representative set of carbapenem-resistant GNB strains to evaluate their contribution to appropriate epidemiological surveillance and therapy of associated infections. A number of 70 Enterobacterales MDR bacterial strains were consecutively isolated from patients with different infections (79 %) and carriers (rectal portages, 21 %) hospitalized at the Fundeni Clinical Institute from November 2017 - April 2018. The strains, previously characterized by PCR, were investigated comparatively by two immunochromatographic tests, NG-Test Carba 5 and RESIST-3 O.K.N., able to detect KPC, OXA-48 like NDM, VIM, IMP, and OXA-48 like, KPC, NDM, respectively. KPC was the main carbapenemase detected (37 %), followed by OXA-48 (30 %). Both rapid immunochromatographic tests demonstrated high sensitivity and specificity, the results being 100 % concordant with the results of the PCR method. The immunochromatographic assay is, therefore, a cheap and reliable method for the rapid detection, within 15 minutes, of carbapenemase-producing strains. Rapid and accurate identification of carbapenemases is significant for clinical and epidemiological purposes, infection control, and antimicrobial therapy's effectiveness.

Keywords: carbapenemase-resistant *Enterobacterales*; NG-Test Carba 5; RESIST-3 0.K.N; immunochromatographic.

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1. Introduction

In Romania, the profile of carbapenem resistance (CR) genes found in multidrug-resistant (MDR) Gram-negative bacilli (GNB) is a major public health problem, both clinically being associated with difficult to treat infections, as well as epidemiologically, due to the risk of nosocomial and community spread of resistant clones [1]. The reporting of an increasing number of CR strains has highlighted the need for a closer phenotypic and molecular

investigation of bacterial resistance, involving rapid screening and confirmatory PCR methods [2]. Resistance to carbapenems can be caused either by enzymatic mechanisms (carbapenems hydrolysis mediated by carbapenemases) or by non-enzymatic mechanisms (acquired by impermeability plus / minus hyperproduction of Amp C) [3]. The ability of carbapenems to hydrolyze all β lactam antibiotics narrowed the spectrum of active antibiotics to very few. The infections caused by these bacteria usually have a poor prognosis, leading to a high mortality rate [4]. Antibiotics such as colistin, tigecycline, ceftazidime-avibactam, and imipenem-relebactam are today considered to belong to the last resort antibiotics for these infections [5,6]. Ceftazidime-avibactam and imipenem-relebactam showed excellent in vitro activity against enterobacterial strains producing extended-spectrum β- lactamases (ESBL) or AmpC, simultaneously with carbapenemases of KPC (*Klebsiella pneumoniae* carbapenemase) or OXA-48 (oxacillinase 48) type [7,8]. Recently, two rapid immunochromatographic tests that detect five of the major clinically important carbapenemases, i.e., KPC, NDM, OXA-48, VIM, and IMP, both from bacterial culture and blood culture, have been developed for diagnostic purposes [9-13]. In this study, the carbapenemase production in the bacterial cultures was investigated in *Enterobacterales* strains that showed resistance to at least one carbapenem, using the two rapid immunochromatographic tests, and the obtained results were compared with those provided by the PCR reference method.

2. Materials and Methods

2.1. Bacterial strains.

A total number of 70 strains of *Enterobacterales*: *Klebsiella (K.) pneumoniae* (n=63), *Providencia stuartii* (n=5), *Escherichia coli* (n=1), and *Serratia marcescens* (n=1) were investigated by rapid immunochromatographic tests, NG-test Carba 5 (*NG Biotech, Guipry, France*) and RESIST-3 O.K.N. (*Coris BioConcept, GemBloux, Belgium*) and confirmed by molecular methods. The non-duplicated strains were isolated during a period of 6 months (November 2017 - April 2018) from patients harboring different infections (urine samples 35 %, bronchial secretions 22 %, other sources 18 %: blood cultures, ascites fluids, abscesses, colonized catheters) and from asymptomatic carriers (digestive colonization) hospitalized in Fundeni Clinical Institute. In the case of 14 patients, the strains were isolated from double sources (bronchial secretion + urine samples, bronchial secretion + wound secretion, blood culture + urine samples, blood culture + rectal swab).

Quality control was performed using *K. pneumoniae* BAA 1705 (KPC positive), *K. pneumoniae* ATCC 700603 (ESBL positive), and *E. coli* ATCC 35218 (ampicillin R, TEM positive, ESBL negative). All strains were identified at the species level using BD Phoenix and Vitek 2 Compact analyzers.

2.2.Phenotypic detection of carbapenemases.

Phenotypic resistance to carbapenems was investigated according to CLSI 2021 (Clinical Laboratory Standard Institute) by disc diffusion and/or automatic method (BD Phoenix / Vitek 2 Compact), depending on the type of sample [14].

In the case of carrier strains, the rectal swabs were inoculated on Brilliance ESBL and CRE chromogenic media (*Oxoid, UK*). Suspected colonies were identified at the genus/species level (BD Phoenix / Vitek 2 Compact) [15]. The phenotypic confirmation for ESBL production was performed by double-disc agar diffusion (Ceftazidime-Amoxicillin / Clavulanic acid-

Ceftriaxone) and CR by disc diffusion imipenem, ertapenem, and meropenem, on Mueller Hinton agar, using the standard recommendations [16]. Subsequently, the confirmed strains were tested for carbapenemase production by the two immunochromatographic assays.

2.3. Immunochromatographic testing with RESIST 3 OKN and NG Carba5.

Rapid immunochromatographic tests contain an independent cassette for different carbapenemase targets: KPC, NDM, VIM, IMP, and OXA-48 (*NG-carba test 5*), respectively OXA-48, KPC, NDM (*RESIST-3 O.K.N.*) and a bacterial cells lysis buffer. The principle of the methods, the workflow, the visual reading, and the interpretation of the results are identical for the two tests. The tests were performed according to the manufacturer's instructions, in parallel, from the same bacterial culture, as follows: 2-3 colonies with the same morphology from the 24 hours culture grown on lactose medium or blood agar were suspended in an Eppendorf tube, in the 10 drops of lysis buffer [17]. After vigorous shaking, approximately 100 µl of the bacterial suspension (3 drops) were added onto the test strip. After 15 minutes from the appearance of the control line - the time in which the suspension migrated completely - the results were read and interpreted. For a positive test result, the presence of a red line in the test area specific to each carbapenemase is recorded [18].

2.4. Detection of carbapenemase genes.

DNA extraction was performed by an adapted alkaline extraction method. Simplex and multiplex PCR (*PCR thermal Corbett thermocycler*) amplifications were performed for bla_{NDM}, bla_{OXA-48}, bla_{KPC}, bla_{VIM}, bla_{IMP} genes using previously described primers [19] in a final volume of 20µL (*PCR Master Mix 2x, Thermo Scientific*), with a content of 1 µl of bacterial DNA, using the following conditions: initial denaturation (95° C, 10 min), followed by 36 cycles of denaturation (94° C, 30 sec), alignment of primers (52° C, 40 sec), extension (72° C, 50 sec) and final extension (72° C, 5 min). DNA fragments were analyzed by electrophoresis in a 1 % agarose gel, migration at 100V for one hour in 1x TAE (40 mmol / L Tris – HCl [pH 8.3], 2 mmol / L acetate, 1 mmol /L EDTA) containing 0.05 mg / L ethidium bromide.

3. Results and Discussion

The general resistance levels recorded for the tested strains ranged from 20 % for amikacin to 100 % for amoxicillin/ clavulanic acid. Remarkably, for all tested antibiotics, except amikacin and ceftazidime/avibactam, the resistance rates were ≥ 79 % (Table 1).

Table 1. The resistance levels (%) to different antibiotics for the tested *Enterobacterales* strains, according to CLSI 2021.

Antibiotics	Non-susceptible isolates (%)
Amoxicillin/ clavulanic acid	100
Ceftazidime	95.83
Ceftriaxone/Cefotaxime	95.83
Ciprofloxacin	91.6
Levofloxacin	91.6
Amikacin	20
Gentamicin	87.5
Piperacillin/tazobactam	87.5
Ertapenem	91.6
Imipenem	91.6
Meropenem	97

Antibiotics	Non-susceptible isolates (%)
Trimethoprim/sulfamethoxazole	79.16
Tigecycline[1]	85.7 (for strains with CMI > 4)
Ceftazidime/avibactam	45.5

[1] Not standardized antibiotic for CLSI 2021

However, the main contributor species to this high resistance rate was *K. pneumoniae*. All *K. pneumoniae* investigated strains showed a high-level resistance rate to all classes of tested antibiotics.

All five *Providencia stuartii* strains were resistant to all classes of tested antibiotics (including aztreonam), thus harboring a pan-drug-resistant (PDR) phenotype [20]. For extensively drug-resistant (XDR) and PDR strains initially tested by disc diffuson by extended antibiogram, the automatic method performed the confirmation of resistance. All strains were resistant to ertapenem, and 95.7 % of the strains (67/ 70) were resistant to all tested carbapenems: ertapenem, imipenem, and meropenem.

Following the PCR amplification and the electropherogram analysis performed in this study (Figure 1), the following results were obtained for the investigated genes: *bla*$_{KPC}$ (37 %), *bla*$_{OXA-48}$ (30 %), *bla*$_{NDM}$ (21.4 %).

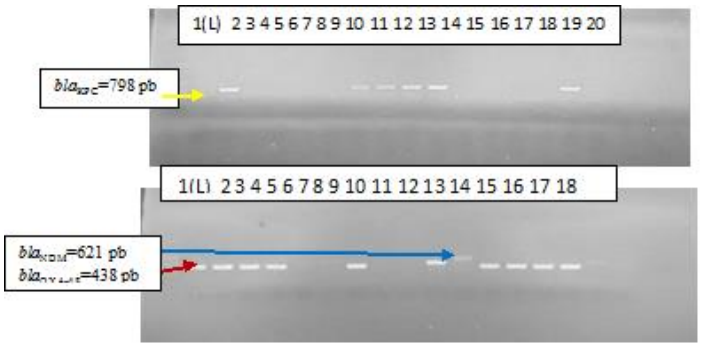

Figure 1. Electropherogram of amplicons obtained for *bla*$_{KPC}$, *bla*$_{NDM}$, and *bla*$_{OXA-48}$ genes in enterobacteria.

The PCR analysis revealed that the *K. pneumoniae* strains harboured the following carbapenemase encoding genes: *bla*$_{KPC}$ (43 %), *bla*$_{OXA-48}$ (30 %), *bla*$_{NDM}$ (19 %), *bla*$_{OXA-48}$ + *bla*$_{NDM}$ (5 %), *bla*$_{KPC}$ + *bla*$_{NDM}$ (3%).

For three strains, no carbapenemase gene was detected, which implies either the presence of a non-enzymatic mechanism or the presence of another less common carbapenemase.

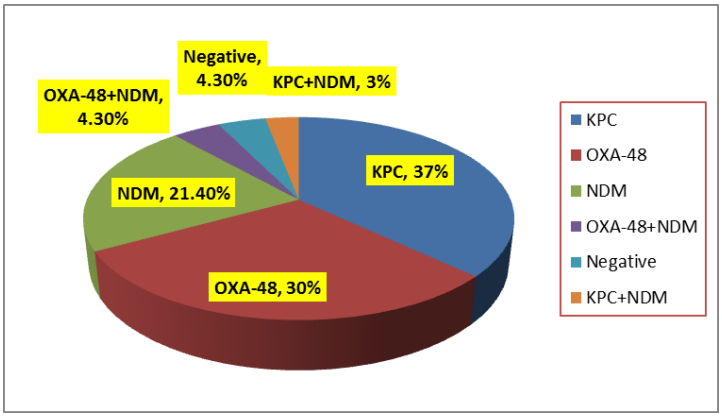

Figure 2. Graphic representation of the incidence of carbapenemase harbored by the tested strains.

The two immunoenzymatic tests NG-test carba 5 / RESIST-3 O.K.N. have detected in the tested Enterobacterales strains the following carbapenemases: KPC (37 %), OXA-48 (30

%), NDM (21.4 %), OXA-48 + NDM (4.3 %), KPC + NDM (3 %) (Figure 2/ Table 2). The obtained results were identical for the two tests (Figure 3), presenting a sensitivity and specificity of 100 % compared to the PCR gold standard.

Table 2. Results of the immunochromatographic tests compared to PCR gold standard.

Bacterial strain	Number of strains	RESIST 3 O.K.N.	NG-Test Carba 5	PCR gene(s)
Klebsiella pneumoniae	26	KPC	KPC	bla_{KPC}
K.pneumoniae BAA 1705		KPC	KPC	bla_{KPC}
	20	OXA-48	OXA-48	bla_{OXA-48}
	12	NDM	NDM	bla_{NDM}
	3	OXA-48+NDM	OXA-48+NDM	bla_{OXA-48}+bla_{NDM}
	2	KPC+NDM	KPC+NDM	bla_{KPC}+bla_{NDM}
K.pneumoniae ATCC 700603		ND[1]	ND	-
Providencia stuarttii	3	NDM	NDM	bla_{NDM}
	2	ND	ND	-
Escherichia coli	1	OXA-48	OXA-48	bla_{OXA-48}
E. coli ATCC 35218		ND	ND	-
Serratia marcescens	1	ND	ND	-

[1]ND-Not Detected.

Figure 3. Examples of positive immunochromatographic tests showing the similarity of the obtained results regarding the detection of carbapenemases: *K. pneumoniae* producing NDM, KPC, and OXA-48 (left), *Providencia stuartii* carbapenemase-negative (middle), *Escherichia coli* producing OXA-48 (right).

Three of the *Providencia stuartii* PDR strains were producing NDM, while for two of them as well as for one *Serratia marcescens* strain, no carbapenemase was detected. The only carbapenemase-positive *Escherichia coli* produced OXA-48. Moreover, a study conducted in 2020 showed that *P. stuartii* carbapenem-resistant strains were confirmed to produce MBL-NDM in 85.42 % of cases, and only one strain was confirmed NDM-VIM in Romania [21].

The great advantage of using immunochromatographic methods is the rapid detection of carbapenemases directly from the culture [22]. The presumptive results of these tests performed directly from the chromogenic media, without phenotypic confirmation, show high sensitivity. Thus, at 24 hours after inoculation of chromogenic media and performing an immunochromatographic test, we can report the presumptive presence of a carbapenemase-producing strain. Subsequently, the strain will be confirmed by the steps provided in the working procedure or by the PCR technique (Figure 2).

On the other hand, NG-carba 5 and RESIST-3 O.K.N. rapid tests can be used in place of other phenotypic assays, such as mCIM, e CIM and CARBA NP [23]. Both mCIM and eCIM detect and differentiate between the two majors groups of carbapenamases, i.e., serine beta-lactamase (ex. KPC and OXA-like) and metallo beta-lactamase (VIM, IMP, NDM). The CARBA NP, another phenotypic assay, has similar limitations to CIM test methods and does not differentiate between the produced carbapenemase [24,25]. Using

immunochromatographic methods, we can both detect and differentiate the carbapenemases in only 15 minutes, without any special training and with minimum costs [26].

There are many studies regarding the performance of these two assays compared with PCR method, high sensitivity and specificity, which confirm their ability to detect carbapenemase-producing *Enterobacterales*. In a study of 186 clinical isolates of carbapenem-resistant *Enterobacterales*, the NG-test Carba 5 detected KPC, NDM, OXA-48-like, IMP, and VIM with a sensitivity and specificity of 100 % and 100 %, respectively. The RESIST-5 O.O.K.N.V. detected KPC, NDM, OXA-48-like, and VIM with a sensitivity and specificity of 99.4 % and 100 %. Also, for reference strains, the sensitivity and specificity of the two tests were 100 % and 100 %, respectively [22].

The same results, with excellent sensitivity and specificity, were shown by El Kettani et al.; the study was focused on 97 *Enterobacterales* strains for diagnostic purposes, with reduced sensitivity to ertapenem and suspicion of carbapenemase production. The results of the RESIST 5 O.O.K.N.V. (OXA-48, NDM, VIM, KPC, and OXA-163) directly from a bacterial colony showed 100 % concordant with the PCR results. They were also able to detect two carbapenemases simultaneously with 100 % sensitivity, and no cross-reaction was noted. In this study, 66 isolates were PCR positive and RESIST O.O.K.N.V. positive, while 27 isolates were PCR and RESIST O.O.K.N.V. negative [27]. In another prospective study on 161 consecutive *Enterobacterales* suspected to produce a carbapenemase, 91 strains were positive, while in 70 strains, no carbapenemases were detected. For both tests, the results were 100 % concordant with the PCR tests [28].

Carbapenemase-producing *Enterobacterales* strains have emerged and spread in Romania since 2010. In our country, little and limited information is known about the production and spread of carbapenemase-producing *Enterobacterales* / *K. pneumoniae* strains and the type of carbapenemase produced. OXA-48 and NDM were reported as the most circulating carbapenemases between 2010-2015 among *Enterobacterales* strains [29, 30, 31, 32, 33]. The first study [19] of a significant number of strains was published in 2014: 79 strains of *Enterobacteriaceae* (currently *Enterobacterales*) and 84 strains of non-fermentative CR, GNB rods were isolated between 2011 and 2012 from Intensive Care patients from two large hospitals in Bucharest. OXA-48 and NDM were the main carbapenemases detected. No KPC was produced by these strains. In another study [30], published in 2015, conducted over six months (November 2013-April 2014) which included 10 major hospitals in Romania, 75 strains of *K. pneumoniae* were analyzed, of which 65 produced carbapenemases. OXA-48 was the main carbapenemase detected (78.5 %), followed by NDM (12.5 %), KPC (6 %) and VIM (3%). OXA-48 was also the main carbapenemase detected (73.77 %) in another study, published in 2018, performed on a number of 61 strains of *Enterobacterales* isolated from patients in an emergency university hospital in Bucharest [32]. In this study, the combination of OXA-48 + NDM was detected in 13.11 % of isolates, followed by KPC (8.19 %) and NDM (3.27 %). The first published data in Romania thus show the high prevalence of OXA-48 carbapenemase. Gradually, the types of carbapenemases have diversified. A recent study [33] on 74 strains of *K. pneumoniae* isolated from 9 hospitals in Romania, performed between January and June 2020, showed that 70 of the investigated strains (94.6%) were positive for the following carbapenem resistance genes: 47 % for OXA-48, 32.5 % for KPC, 11.42 % for NDM, while 6 strains (8.57 %) were producing two carbapenemases (KPC + NDM and OXA-48 + NDM). A high percentage (58 %) of these strains were resistant to colistin, all of which were negative for the mcr1-5 genes. The *K. pneumoniae* strains isolated between 2011-2012

were not positive for KPC-type carbapenemases [19]. However, the latest published data showed an increased incidence of KPC-producing strains starting in 2018 and the circulation of *K. pneumoniae* strains harboring two types of carbapenemases, similar to our study. More recently, there have demonstrated a high reservoir of antibiotic resistance and virulence markers in *K. pneumoniae* ST101 isolated from intra-hospital infections and wastewater in south Romania; the transmission of MDR, carbapenemase, and ESBL-producing *K. pneumoniae* ST101 from hospital to hospital effluent and its persistence after the chlorine treatment suggesting its dissemination in the community and also in different aquatic compartments [34,35].

4. Conclusions

The two rapid immunochromatographic diagnostic tests, NG-Test Carba 5 and RESIST-3 O.K.N. are useful for the early and accurate detection of the most frequent carbapenemases. Overall, the speed and ease of use of the OKN assay represent significant technical advances. This method's rapid detection and identification of carbapenemases allow clinical laboratories to help prevent spreading and control infections with carbapenemase-producing isolates in healthcare facilities. At the same time, to use the newly developed antimicrobials and ensure their clinical effectiveness, it is necessary to know the circulating carbapenemase.

Funding

This research was funded by The Executive Agency for Higher Education, Research, Development and Innovation Funding (UEFISCDI), grant number PN-III-P4-ID-PCCF-2016-0114 and PN-III-P1-1.1-TE-2021-1515 (TE 112/2022) and the APC was funded by the Ministry of Research, Innovation, and Digitalization through Program 1—Development of the national R&D system, Subprogram 1.2—Institutional performance—Financing projects for excellence in RDI, C1.2.PFE-CDI.2021-587 Contract no. 41 PFE/30.12.2021. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

Acknowledgments

The financial support of PN-III-P4-ID-PCCF-2016-0114; PN-III-P1-1.1-TE-2021-1515 (TE 112/2022) and C1.2.PFE-CDI.2021-587 Contract no. 41 PFE/30.12.2021 is gratefully acknowledged.

Conflicts of Interest

The authors declare no conflict of interest.

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